Polyol-producing lactic acid bacteria isolated from sourdough and their application to reduce sugar in a quinoa-based milk substitute

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Abstract

The interest for plant-based dairy substitutes is expanding rapidly and consumers are opting for nutritious and healthy dairy alternatives. The reduction of sugar using different exogenous enzymes in combination with lactic acid fermentation in a quinoa-based milk substitute was explored in this study. Different amylolytic enzymes were used to release sugar from the raw material, which were further metabolised to mannitol, due to fermentation with two heterofermentative lactic acid bacteria. Using these two biotechnological techniques enables the reduction of sugar, while also preserving some of the sweetness. *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 were isolated from sourdough. Both strains showed high viable cell counts with *L.~~euconostoc~~ citreum* TR116 > 8.4 and *L.~~actobacillus~~ brevis* TR055 > 9.3 log cfu/ml, and a reduction in pH to 3.7 and 3.5 respectively. When fructose was available, mannitol was produced in conjunction with acetic acid in addition to lactic acid. Due to these processes, the original glucose value was reduced from 50 mmol/100g to approximately 30 mmol/100g, which equates to a glucose reduction of 40%. In respect to mannitol production, both strains performed well: *L.~~euconostoc~~ citreum* TR116 showed a conversion factor of 1:1 from fructose to mannitol, while *L.~~actobacillus~~ brevis* TR055 showed a lower yield, with a conversion factor of 1:0.8. Glycaemic load was reduced by more than a third, bringing it down to the low range with a value of about 10. Overall, enzymatic modification in conjunction with mannitol-producing lactic acid bacteria shows great potential for further possible application in the development of nutritious and sugar reduced plant-based milk substitutes.

Keywords
Milk substitute, Lactic acid bacteria, Quinoa, Sugar reduction, Mannitol, Glycaemic index

1. Introduction

Plant-based milk substitutes (PBMSs) are gaining popularity and the interest is expanding rapidly. Consumers are choosing dairy substitutes over dairy products for various reasons; obviously so in the case of individuals suffering from milk allergies and intolerance, but an increasing consumption is based on preference. In this regard, PBMSs can serve as a sustainable, ethical and nutritious option to meet the needs of consumers. Owing to this increasing interest, the market is expected to grow at a significant rate: MarketsandMarkets (2017) estimated the value of the dairy alternative market to be 7.37 Billion USD for 2016 and predicted a growth rate of 11.7% from 2017 on, reaching a forecasted market value of 14.36 Billion USD in 2022. Nevertheless, many studies reported several concerns about the nutritional value of some products (Jeske et al., 2017; Katz et al., 2005; Sousa et al., 2017). In particular, the low protein content was found to be a major risk. Furthermore, PBMSs based on starchy raw materials, such as rice or quinoa contain high amounts of sugar due to hydrolysis of starch and release of maltose and/or glucose thereof. Sugar contents and *in-vitro* glycaemic indices of commercial PBMSs were analysed, and rice-, and coconut-based products especially showed high values for the glycaemic indices with 97.74, and 96.82, respectively, with sugar content of 7.02 and 1.86 g/100g, respectively (Jeske et al., 2017). High sugar consumption affects human health, being a major inducer for obesity and chronic diseases (Lustig et al., 2012). The public awareness of this problem is increasing and consumer behaviour is changing: 64% of consumers on the Island of Ireland are concerned about their sugar intake (James Wilson, 2018), and similarly German consumers have reduced their sugar consumption by 48% (Mintel Press Team, 2017). For this reason, research and industry are investigating methods for sugar-reduction or use of sweeteners as alternatives to sugar. Mannitol, as one promising alternative, is a natural sugar alcohol, prevalent in several plants, fungi, yeast and bacteria (Wisselink et al., 2002). It has a sweet taste, being perceived about 40% less sweet than sucrose, and its incorporation in food has several beneficial effects; these include health claims

Abbreviation:
PBMS: Plant-based milk substitutes
QBMS: Quinoa-based milk substitute

relating to protection against tooth-decay and reduction of the glycaemic response, both due to mannitol not being absorbed in the human intestine, and thus exhibiting a low calorific value. Both claims are approved by the European Food Safety Authority (2011). Although an increasing amount of consumers (17% in Germany) believe that plant-based yoghurt alternatives are healthier, the biggest challenge lies in the taste for these products (Mintel Press Team, 2017). In this regard, mannitol could improve the properties of these products, increasing both health benefits and flavour at the same time. Industrially, mannitol is produced by catalytic hydrogenation of a glucose/fructose syrup, producing a mixture of sorbitol and mannitol. However, the yield is low and costs are high for this chemical process (Grembecka, 2015). As an alternative, lactic acid bacteria (LAB) can be used to produce mannitol in a more sustainable and efficient way. Heterofermentative LAB can reduce fructose directly to mannitol. It is catalyzed by the enzyme mannitol dehydrogenase and, metabolically, serves to regenerate NAD^+ (Wisselink et al., 2002). Fermented foods are attracting increased interest and recently much emphasis has been granted to their unique functional properties and contribution to the health of consumers. Their application has evolved from preserving food to understanding and exploiting metabolites, other than organic acids and antifungal compounds. Studies focus on compounds associated with health benefits and additional functional properties, such as mannitol or exopolysaccharides (Chilton et al., 2015; Lynch et al., 2018; Selhub et al., 2014; Tamang et al., 2016). Further, new raw materials are explored as substrate for LAB fermentation and for the development of novel products. For instance, the ancient pseudocereal quinoa has received renewed interest, particularly in Western countries due to its high nutritional value (Arendt and Zannini, 2013). It is especially rich in protein and essential amino acids, contains adequate levels of important micronutrients such as minerals and vitamins, and significant amounts of other bioactive compounds, such as polyphenols (Alvarez-Jubete et al., 2010; Arendt and Zannini, 2013). As a versatile substrate, quinoa has been used for different fermented products; Axel et al. (2015) improved the nutritional value and bread quality using quinoa flour as a base for sourdough with exceptionally high amounts of antifungal compounds; Zannini et al. (2018) developed a quinoa-based yoghurt, having a higher water holding capacity and viscosity than a chemically-acidified control, due to dextran expolysaccharide excretion by an LAB strain. In addition, fermentation has been shown for many cereals and legumes to

improve sensorial and textural properties (Peyer et al., 2016) and could be used as a tool to ameliorate grassy and bitter off-flavours, characteristic for quinoa.

In this study, a quinoa based milk substitute was used as a substrate to study the production of mannitol by means of a two-step process including enzyme treatment, and fermentation with two heterofermentative LABs. The samples were treated with amylases and a glucose-isomerase, in order to generate fructose as a substrate, which was further metabolized to mannitol by the action of LAB. The aim was to study the potential for sugar-reduction using this two-step process.

2. Materials and methods

2.1 Materials, strains and culture conditions

Organic quinoa was obtained from Ziegler & Co. GmbH Naturprodukte (Wunsiedel, Germany). Chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise stated. The microorganisms *Leuconostoc citreum* TR116 and *Lactobacillus brevis* TR055 were isolated from yellow pea sourdough and teff sourdough, respectively, and belong to the culture collection of the Department of Biological Sciences, Cork Institute of Technology, Ireland. These cultures were selectively chosen for their ability to produce mannitol. The LAB isolates were maintained as frozen stocks in 40% (w/w) glycerol at -80°C. The strains were routinely sub-cultured on de Man Rogosa and Sharp (MRS) agar under anaerobic conditions for 24 h at 30 °C.

2.2 Preparation of quinoa-based milk substitute

50 g organic quinoa flour and 350 g water were mixed in a semi-industrial blender (Kenwood Major Titanium, New Lane, Havant, UK) at maximum speed for 3 minutes. To each sample 250 mg α-amylase (Hitempase 2XP, Kerry, Carrigaline, Ireland), 300 µL amyloglucosidase (Attenzuzyme, Novozymes), and 36.6 µL protease (Flavourzyme, Novozymes) were added. The samples were mixed again for 30 s at lower speed. Additionally, 0.8 g glucose-isomerase were added to some of the samples (labelled as "iso"). All samples were kept in a stirring water bath at 60 °C for 24 h for enzyme action and cooled to 25 °C within 20 min (Lochner mashing device LP electronic, Berching, Germany). Samples were cooled on ice straight after, filtered with cheese clothes and homogenized

(APV Homogenizer, SPX FLOW, Inc., Charlotte, USA) at 150 bar for the 1st stage, 30 bar for the 2nd stage. Finally, the samples were pasteurized in a water bath at 65 °C for 30 min.

2.3 Fermentation

Single colonies of each LAB strain were propagated twice in 10 mL MRS broth in anaerobic and static conditions for 24 h at 30 °C. Cultures were cultivated until the late exponential phase (ca. 14 h) and enumerated by performing a viable plate count in duplicate. After cell count determination, suspensions were prepared in the same manner for inoculation and harvested by centrifugation at 9000 g for 10 min at 4 °C and washed twice with Ringer's solution. The inoculation was performed at 7 log cfu/mL directly into tempered quinoa-based milk substitute (QBMS) samples. Fermentation was performed anaerobically, under static conditions at 30 °C for 24 h. Figure 1 depicts the enzymatic processing of quinoa starch, using the exogenous enzymes (α-amylase, γ-amylase, and glucose-isomerase) added at the before outlined part of the preparation of QBMS samples, and the endogenous enzyme mannitol-dehydrogenase, which is secreted by *Leuconostoc citreum* TR116 and *Lactobacillus brevis* TR055 during the fermentation test.

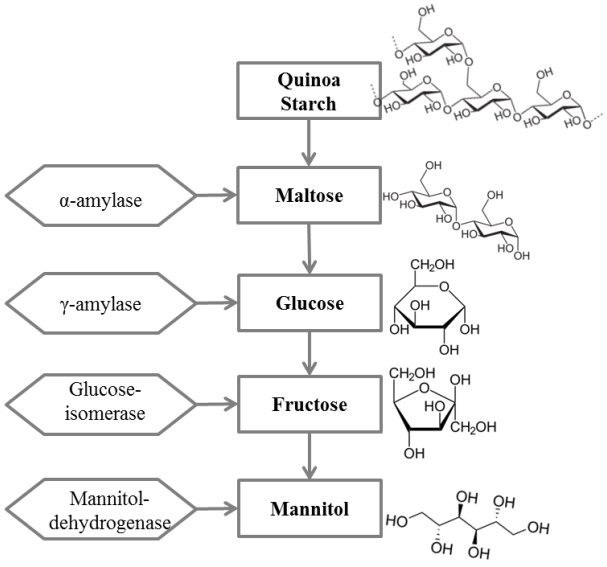

Figure 1 Enzymatic processing of quinoa starch with exogenous enzymes (α-amylase, γ-amylase, and glucose-isomerase), and endogenous enzymes, secreted by LAB (mannitol-dehydrogenase)

2.4 Compositional analysis

Compositional analyses were performed on the quinoa flour and unfermented samples. Total nitrogen content was determined according to the Kjeldahl method (MEBAK 1.5.2.1). Nitrogen content was converted into protein using the factor 5.75 according to Fujihara et al. (2008). Fat content was measured following the Soxhlet method. Ash content was determined in a muffle furnace by incineration (4 h, 600 °C), pre-heated in crucibles (1 h, 100 °C). The moisture content was determined by drying in an oven at 103 °C until constant mass was reached. Total starch was analysed using the enzyme kit K-TSTA supplied by Megazyme, Ireland.

2.5 Viable cell counts

Total cell counts of LAB were performed on MRS agar plates after incubation for 48 h under anaerobic conditions using Anaerocult A gas packs (Merck, Darmstadt, Germany) at 30 °C.

2.6 Measurement of titratable acidity, and pH

The total titratable acidity (TTA) was determined by suspending 5 g of sample in 45 mL distilled water and titrating against 0.1 N NaOH to pH 8.5 (Katina et al., 2006). After 3 min, the pH was readjusted to 8.5. The TTA was expressed as the number of millilitres of NaOH used for titration. The pH was monitored using a commercial digital pH meter.

2.7 Determination of sugar and organic acids profiles

~~For sugar analysis, s~~Samples were diluted with water, ~~and~~ filtered (0.2 µm)~~. Sugar profiles were~~ and analysed by high performance liquid chromatography using an Agilent Infinity 1260 HPLC System. For sugar analysis the system was equipped with a Waters Sugar-Pak, 300 x 6.5 mm HPLC column at 0.5 mL/min flow rate of 0.0001 mmol/L CaEDTA at 80 °C, ~~and detected by~~using a refractive index detector (Agilent Technologies, Palo Alto, CA) for detection. Glucose, maltose, fructose, and mannitol were used as external standards. Results were reported in mmol/100g QBMS. Organic acids were determined using an Agilent Hi-Plex H, 7.7 x 300 mm, pack size 8 µm HPLC column with a 178 PL Hi-Plex Guard column mounted upstream at a flow rate of 0.5 mL/min of 0.005 mmol/L H_2SO_4, and a column temperature of 60 °C. Lactic acid, and acetic acid were used as external standards. Results were reported in mmol/100g QBMS.

~~***2.8 Determination of organic acids***~~

~~Organic acids were determined by high performance liquid chromatography using an Agilent Infinity 1260 HPLC System equipped with a diode array detector (Agilent Technologies, Palo Alto, CA). All measurements were performed using an Agilent Hi-Plex H, 7.7 x 300 mm, pack size 8 µm HPLC column with a 178 PL Hi-Plex Guard column mounted upstream. Samples were previously sterile filtered through (0.2 µm) and analysed at a flow rate of 0.5 mL/min of 0.005 mmol/L H_2SO_4, at a column temperature of 60 °C. Lactic acid, and acetic acid were used as external standards. Results were reported in mmol/100g QBMS.~~

2.9 Glycaemic index

In vitro determination of the glycaemic index (GI) was evaluated according to Magaletta & DiCataldo (2009) using a calculation designed by an artificial neural network. A certain amount of sample (equivalent to 0.5 g of available carbohydrates, based on the results of sugar and starch analysis) was digested by a multi-enzyme preparation. The digestate was analysed for glucose, fructose, lactose, galactose, and maltitol with HPLC, described as above. These results, together with the results from the protein and fat determination, were used to feed the calculation:

$$GI = 26.264529 - 1.048186 \cdot Protein\ [\%] - 0.248138 \cdot Fat\ [\%]$$

$$+621.7824 \cdot Glucose\ [\%] - 52.7993 \cdot Fructose\ [\%]$$

$$-233.67679 \cdot Lactose\ [\%] - 61.21071 \cdot Galactose\ [\%] - 84.689245\ Maltitol[\%]$$

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Glycaemic load (GL) was calculated according to Atkinson et al. (Atkinson et al., 2008):

$$GL = (GI \cdot available\ carbohydrate\ (g)\ per\ portion)/100$$

The portion size was set to 250 g.

2.10 Physicochemical Properties

Rheological behaviour of the products was characterised using a controlled stress rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a sensor system of coaxial cylinders (C-CC27-T200/SS, Anton Paar GmbH, Austria). The shear stress (σ) was measured as a function of shear rate (y) ranging from 0.5 to 100 s^{-1} within 500 s. The measurements were carried out at 10 °C. The apparent viscosity measured at 10 s^{-1} is referred to as viscosity. Stability was determined through phase separation analysis using an analytical centrifuge (LUMiSizer; LUM GmbH, Berlin, Germany). The instrumental parameters used were as follows: 1000 rpm for 30 min followed by 3000 rpm for 60 min at 24 °C. Separation rate in %/h was determined by plotting the % of transmission over the time. Syneresis of quinoa milk was analysed using a slight modification of the centrifugation method previously reported by Keogh and O'Kennedy (1998). 40 g of sample were centrifuged at 220 g for 10 min at 4 °C. The supernatant was poured off and weighed again. Syneresis was expressed as a %. Colour values were measured using the CIE L*a*b* colour system and obtained using illuminant D65. The instrument used was a colorimeter (CR-400, Konica Minolta, Osaka, Japan). Colour of samples was characterised according to whiteness index (WI), defined as:

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

2.11 Statistics

All analyses were carried out at least in triplicate. Means were compared with one-way analysis of variance (ANOVA) and Tukey pairwise. The significance level was set to $\alpha = 0.05$.

3. Results and discussion

3.1 Compositional analysis

The composition of the quinoa flour used for the preparation of the samples given in % (w/w) was as follows: moisture: 9.6% ± 0.4, protein: 14.64% ± 0.14, ash: 2.59% ± 0.03, fat: 7.24% ± 0.00, and total starch (dry basis): 67.4% ± 3.55. The prepared QBMS samples contained 0.84% ± 0.01 protein, 0.29% ± 0.01 ash, 0.33% ± 0.04 fat and <1.8 mg/L starch. Due to hydrolysis of starch, the samples contained glucose with 9.09% ± 0.45. The level of fructose during glucose-isomerase treatment at different time

points is displayed in figure 1. Fructose contents continued to increase, while finally the amount of glucose was reduced to 7.21% ± 0.09 and fructose levels occurred at 1.55% ± 0.07 after 24 hours.

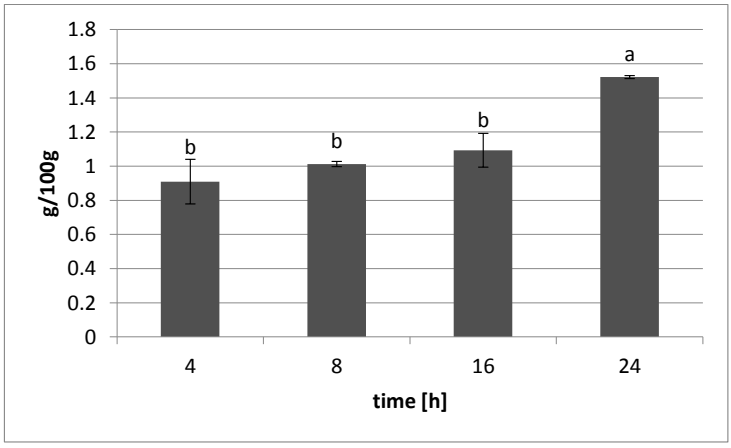

Figure 1~~2~~ Concentration of fructose in quinoa-based milk substitutes over time during glucose-isomerase treatment. Values that share a label are not significantly different from one another ($p < 0.05$).

3.2 Cell g~~G~~rowth and acidification properties~~kinetics~~

The values of viable cell counts, pH, total titratable acidity, and acid profile are presented in Table 1. The results showed that QBMS facilitated the growth of *L. citreum* TR116, as well as *L. brevis* TR055. The latter showed a more vigorous growth, reaching values of 9.35 log cfu/mL, while *L. citreum* TR116 reached cell counts of 8.48 log cfu/mL after 24h incubation. Ruiz Rodríguez et. al (2016) found both strains to be autochthonous in spontaneously fermented sourdough produced from quinoa. Both strains showed similar cell counts in QBMS, regardless of treatment with isomerase, i.e. the presence of fructose in the media had no impact on cell growth. However, the presence of fructose influenced acid production; TTA values increased for both strains in samples due to the treatment with glucose-isomerase, from 4.38 to 6.79 mL for *L. citreum* TR116, and from 6.04 to 8.68 mL for *L. brevis* TR055. The pH, however, dropped to 3.75 and 3.52, for *L. citreum* TR116 and *L. brevis* TR055, respectively and was not decreased considerably in QBMS treated with glucose-isomerase. A closer look at the acid profile revealed that in the absence of fructose, only lactic acid was produced (5.49 and 8.82 mmol/100g from *L. citreum* TR116, and *L. brevis* TR055, respectively), while in the

presence of fructose, acetic acid was additionally produced from both strains (4.79 and 4.12 mmol/100g from *L. citreum* TR116 and *L. brevis* TR055, respectively).

Table 1 Cell counts, pH, TTA, and organic acid values of unfermented (Unf.), isomerase treated samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 ~~fermented~~ of quinoa-based milk substitutes.

	cfu [log cfu/mL]	pH	TTA [mL]	Lactic acid [mmol/100g]	Acetic acid [mmol/100g]
Unf.	n.d.	5.37±0.08^{b}	1.70±0.01^{e}	n.d.	n.d.
Unf. Iso	n.d.	5.67±0.05^{a}	1.63±0.06^{e}	n.d.	n.d.
TR116	8.48±0.03^{b}	3.75±0.02^{c}	4.38±0.35^{d}	5.49±0.42^{b}	n.d.
TR116 Iso	8.42±0.26^{b}	3.64±0.03^{cd}	6.79±0.47^{b}	5.59±0.37^{b}	4.79±0.41^{a}
TR055	9.35±0.10^{a}	3.52±0.05^{de}	6.04±0.12^{c}	8.82±0.13^{a}	n.d.
TR055 Iso	9.24±0.09^{a}	3.45±0.02^{de}	8.68±0.27^{a}	8.95±0.08^{a}	4.12±0.23^{a}

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$); n.d. detectable, limit for cfu < 3 log cfu/mL, for lactic and acetic acid < 1mM/100g

Heterofermentative LAB can generate additional ATP with the production of acetic acid from acetyl phosphate. However, this is only possible in the presence of fructose, which acts as an alternative electron acceptor, NAD^{+} is regenerated via the reduction of fructose to mannitol, which would otherwise happen through the production of ethanol from acetyl phosphate (Wisselink et al., 2002).

Table 2 shows the sugar composition with some stoichiometric parameters related to the sugar metabolism. Due to the glucose-isomerase treatment, 8.58 mmol/100g fructose were produced from glucose. Furthermore, glucose was metabolised by both strains and approximately 9 mmol/100g glucose were consumed in all fermented samples; neither the glucose-isomerase treatment and changing carbohydrate composition, nor the bacteria itself had a considerable impact on this value. When fructose was present, both LAB produced mannitol additionally. *L. citreum* TR116 produced 8.58 mM/100g, while *L. brevis* TR055 produced less mannitol, at 7.18 mM/100g. *L. citreum* TR116 metabolized fructose completely to mannitol, with a yield of 100%, while *L. brevis* TR055 achieved a yield of 84%. It was demonstrated previously that heterofermentative LAB can reduce fructose to mannitol with yields of up to 100%, when glucose and fructose where available (1:2) (Wisselink et al.,

2002). *L. citreum* is known to produce mannitol, however other studies have found lower yield values of 89.3% or 70% from fructose (Carvalheiro et al., 2011; Otgonbayar et al., 2011). In these studies, the ratio of fructose to glucose was higher, and the strains used were grown in a different medium. On the other hand, *L. brevis* TR055 showed a lower ratio of fructose to mannitol with about 84%. Therefore, some fructose must have entered the phosphoketolase pathway instead of being metabolized to mannitol. Due to the combined reactions of glucose-isomerase and fermentation, a total amount of about 20 mmol/L glucose were removed and transformed into metabolites like organic acids, mannitol and other compounds. This bioprocess can be used to generate sour, fermented products, while at the same time not losing too much sweetness, since mannitol is produced. Glucose was reduced by approximately 40% (equivalent to 20 mmol/100g) through the action of the glucose-isomerase treatment and being used as a carbon source for bacterial growth. However, the sweetness of the product was only reduced by about 24% and 28% for samples treated with glucose-isomerase and fermented with *L. citreum* TR116 or *L. brevis* TR055, respectively, when considering literature values of the relative sweetness of glucose and mannitol (0.7 and 0.6, respectively, compared to sucrose) (Nutrients Review, 2016).~~the reduced glucose content and the different sweetness levels of glucose and mannitol (0.7 and 0.6, respectively when compared to sucrose) (Nutrients Review, 2016).~~

Table 2 Sugar composition and stoichiometric parameters of unfermented (Unf.), isomerase treated samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 of quinoa-based milk substitutes.~~*Leuconostoc citreum* TR116 and *Lactobacillus brevis* TR055 fermented quinoa based milk substitutes.~~

	Glucose [mmol/100g]	**Fructose [mmol/100g]**	**Maltose [mmol/100g]**	**Mannitol [mmol/100g]**	**Reduction of glucose [mmol/100g]**	**Mannitol yield on fructose**
Unf.	50.44±2.49^{a}	n.d.	1.09±0.13^{a}	n.d.	-$^{-}$	-$^{-}$
Unf. Iso	39.99±0.48^{b}	8.58±0.40^{a}	0.86±0.07^{bc}	n.d.	10.44±2.27^{b}	-
TR116	39.92±2.00^{b}	n.d.	0.99±0.04^{ab}	n.d.	10.52±2.24^{b}	-
TR116 Iso	31.01±0.32^{c}	n.d.	0.67±0.04^{c}	8.58±0.10^{a}	19.43±2.29^{a}	100±5^{a}
TR055	42.38±2.58^{b}	n.d.	0.94±0.08^{ab}	n.d.	8.05±0.22^{b}	-
TR055 Iso	30.14±1.95^{c}	0.17±0.08^{b}	0.72±0.07^{c}	7.18±0.46^{b}	20.3±2.44^{a}	84±2^{b}

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$); n.d., not detectable, < 0.5mM/100g

Furthermore, the glycaemic effect of the samples was determined with an *in-vitro* method. The digestion of carbohydrate-containing food products affects blood glucose levels, also known as the postprandial glycaemic effect. The GI is related to the type of carbohydrates and dependent on the rate of digestion (Wolever et al., 2008). In fact, only glucose can be absorbed directly by the small intestine and used for energy generation; other sugars, such as fructose and galactose must be metabolised by the liver to glucose, or, in the case of sucrose, and most polysaccharides, must be hydrolysed into their constituent monosaccharides before being metabolised further. Therefore, the postprandial rise in blood glucose levels is lower for those carbohydrates. In the case of mannitol, or other non-glycaemic carbohydrates such as dietary fibre and resistant starch, no effect on the blood glucose level can be observed, since these are not digested in the small intestine (Östman et al., 2002) . The GI of all samples was high, ranging from 64 to 76. Only the unfermented, glucose-isomerase-treated sample had a slightly lower GI, due to the conversation from glucose to fructose, which has a lower impact on the blood sugar level (Foster-Powell et al., 2002). Considering the GL on the other hand, considerable differences were observed. The GL relates the GI to a portion size, representing both quality and quantity of carbohydrates being consumed (Barclay et al., 2008). Hence, results represent the impact on the blood sugar level after consuming 250 mL of sample. For the untreated sample, a GL of 16.22 was determined. With fermentation, only a slight, insignificant, reduction was obtained, to 14.40 and 14.09 for *L. citreum* TR116 and *L. brevis* TR055, respectively. A remarkable reduction of more than a third was obtained for both the glucose-isomerase-treated, fermented samples, bringing the GL down to almost the low range (<10) (Venn and Green, 2007). *L. citreum* TR116 showed a value of 10.80 and *L. brevis* TR055 one of 10.43.

Table 3 *In-vitro* glycaemic index, load, and reduction of glycaemic load of unfermented (Unf.), isomerase treated samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 of quinoa-based milk substitutes.~~for *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 fermented quinoa-based milk substitutes.~~

	Glycaemic index [-]	Glycaemic load [-]	Reduction of Glycaemic load [%]
Unf.	69.47±1.37^{bc}	16.22±0.70^{a}	-
Unf. Iso	64.57±1.11^{c}	14.12±0.51^{a}	9.89±1.90^{b}
TR116	76.51±0.22^{a}	14.40±0.72^{a}	14.68±4.08^{b}
TR116 Iso	73.04±1.61^{a}	10.80±0.22^{a}	34.45±2.91^{a}
TR055	70.82±0.60^{abc}	14.09±0.87^{b}	13.15±2.38^{b}
TR055 Iso	72.08±3.95^{ab}	10.43±0.58^{b}	36.71±0.64^{a}

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$)

The depletion of about 20 mmol/100g glucose and ultimate bioconversion into the non-glycaemic carbohydrate, mannitol, resulted in this substantial reduction of GL. Research studies strongly indicate a correlation between high GI and GL and increased risk of type 2 diabetes, breast cancer, gallbladder disease and heart disease, while low GI and GL diets show many health benefits i.e. weight control, protection against colon and breast cancer, obesity, cardiovascular disease, and diabetes (Brand-Miller, McMillan-Price, et al. 2009).

3.3 Physicochemical properties

Samples were analysed for physicochemical properties to asses their characteristics as a beverage. The results are shown in table 4. Due to the drop in pH during fermentation, the samples were destabilized, which is evident in the results for syneresis and separation rate; both values increased from 10.20 to 15.21% and from 13.34 to 21.86 %/h, respectively, due to fermentation of samples with *L. brevis* TR055. No considerable differences between the fermented samples were found. The pH after fermentation is close to the isoelectric point of quinoa proteins, being around 4 (Elsohaimy et al., 2015), resulting in a low solubility and destabilisation. As seen in the measurements of rheology, the decrease of pH did not affect the viscosity and no significant differences were found between samples. Unlike other proteins, such as casein in bovine milk, quinoa proteins do not facilitate a network-forming matrix and gel strength is weak, as shown also by Mäkinen et al. (2014). However, in order to compensate for the lack of network forming properties of the quinoa proteins, Zannini et al. (2018) used an EPS-producing culture (*Weissella cibaria* MG1) to produce a quinoa-based yoghurt substitute.

The resulting yoghurt showed increased viscosity (> 0.5 Pa s), and improved water holding capacity, both due to the amounts of EPS produced. A combined fermentation with an EPS-producing strain could therefore overcome the rheological challenges, generating a product with multiple new functional properties. The samples showed very slight differences for the chromaticity and similar whiteness indices, ranging from 49.49 to 54.32. These values indicate a lower whiteness of the samples compared to bovine milk (81.89), but the values are similar to other commercial PBMS (Jeske et al., 2017).

Table 4 Physicochemical properties of products of unfermented (Unf.), isomerase treated samples (Iso) and fermented samples with *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 of quinoa-based milk substitutes.~~for *Leuconostoc citreum* TR116, and *Lactobacillus brevis* TR055 fermented quinoa-based milk substitutes.~~

	Separation rate [%/h]	Viscosity [mPa·s]	Syneresis [%]	Whiteness Index [-]
Unf.	13.34±1.08^{c}	5.92±0.89^{b}	10.20±1.86^{b}	49.49±1.57^{d}
Unf. Iso	15.04±0.82^{bc}	5.83±0.48^{b}	11.68±1.96^{b}	51.18±1.23^{cd}
TR116	18.25±2.53^{ab}	5.99±1.04^{b}	15.13±1.60^{a}	52.06±1.43^{bc}
TR116 Iso	18.19±1.41^{ab}	8.14±1.41^{a}	16.20±1.05^{a}	53.25±1.94^{ab}
TR055	21.86±2.14^{a}	6.36±1.20^{a}	15.21±1.56^{a}	53.53±0.77^{ab}
TR055 Iso	15.04±1.02^{c}	7.06±0.81^{a}	16.50±0.63^{a}	54.32±1.50^{a}

Values within a column that share a superscript are not significantly different from one another ($p < 0.05$)

4. Conclusion

This study demonstrates a novel biotechnological processing approach to improve nutritional properties and meet consumer demands of PBMSs. The production of mannitol was examined in a quinoa-based milk substitute, using two LAB as starter cultures. It was shown that quinoa serves as a good substrate, facilitating the growth of *L. citreum* TR116 and *L. brevis* TR055 with high viability. The hydrolysis of starch, further conversation of glucose to fructose through the enzyme glucose-isomerase, and subsequent reduction of fructose to mannitol via fermentation, reduced the glucose content by 40% and GL by 35%. *L. citreum* TR116 and *L. brevis* TR055 could be used as novel

functional starter cultures and this approach can be transferred to any kind of fermented food product, such as sourdough or beverages. This laboratory prototype could represent an example of novel PBMSs, characterised by improved nutritional and functional properties and also by a lower carbon and water footprint when compared to their dairy counterparts.

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List of References

Alvarez-Jubete, L., Wijngaard, H., Arendt, E.K., Gallagher, E., 2010. Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. Food Chem. 119, 770–778. https://doi.org/10.1016/j.foodchem.2009.07.032

Arendt, E.K., Zannini, E., 2013. Cereal grains for the food and beverage industries, Journal of Chemical Information and Modeling. https://doi.org/10.1017/CBO9781107415324.004

Atkinson, F., Foster-Powell, K., Brand-Miller, J.C., 2008. Glycemic Load Values : 2008. Diabetes Care 31, 2281–2283. https://doi.org/10.2337/dc08-1239.J.B.M.

Axel, C., Röcker, B., Brosnan, B., Zannini, E., Furey, A., Coffey, A., Arendt, E.K., 2015. Application of Lactobacillus amylovorus DSM19280 in gluten-free sourdough bread to improve the microbial shelf life. Food Microbiol. 47, 36–44. https://doi.org/10.1016/J.FM.2014.10.005

Barclay, A.W., Petocz, P., McMillan-Price, J., Flood, V.M., Prvan, T., Mitchell, P., Brand-Miller, J.C., 2008. Glycemic index, glycemic load, and chronic disease risk—a meta-analysis of observational studies. Am. J. Clin. Nutr. 87, 627–637. https://doi.org/10.1093/ajcn/87.3.627

Carvalheiro, F., Moniz, P., Duarte, L.C., Esteves, M.P., Gírio, F.M., 2011. Mannitol production by lactic

347 acid bacteria grown in supplemented carob syrup. J. Ind. Microbiol. Biotechnol. 38, 221–227.
348 https://doi.org/10.1007/s10295-010-0823-5

349 Chilton, S., Burton, J., Reid, G., 2015. Inclusion of Fermented Foods in Food Guides around the World.
350 Nutrients 7, 390–404. https://doi.org/10.3390/nu7010390

351 Elsohaimy, S.A., Refaay, T.M., Zaytoun, M.A.M., 2015. Physicochemical and functional properties of
352 quinoa protein isolate. Ann. Agric. Sci. https://doi.org/10.1016/j.aoas.2015.10.007

353 European Food Safety Authority, 2011. Scientific Opinion on the substantiation of health claims
354 related to the sugar replacers xylitol , sorbitol , mannitol , maltitol , lactitol , isomalt , erythritol ,
355 D-tagatose , isomaltulose , sucralose and polydextrose and maintenance of tooth mineralis.
356 EFSA J. 9 (4), 1–25. https://doi.org/10.2903/j.efsa.2011.2076.

357 Foster-Powell, K., Holt, S.H. a, Brand-Miller, J.C., 2002. International table of gylcemic index and
358 glycemic load values: 2002. Am. J. Clin. Nutr. 76, 5–56.

359 Fujihara, S., Sasaki, H., Aoyagi, Y., Sugahara, T., 2008. Nitrogen-to-Protein Conversion Factors for
360 Some Cereal Products in Japan. J. Food Sci. 73, C204–C209. https://doi.org/10.1111/j.1750-
361 3841.2008.00665.x

362 Grembecka, M., 2015. Sugar alcohols—their role in the modern world of sweeteners: a review. Eur.
363 Food Res. Technol. 241, 1–14. https://doi.org/10.1007/s00217-015-2437-7

364 James Wilson, M.I., 2018. The Irish drinks industry braces itself for sugar tax | Mintel.com [WWW
365 Document]. URL http://www.mintel.com/blog/drink-market-news/the-irish-drinks-industry-
366 braces-itself-for-the-sugar-tax (accessed 3.19.18).

367 Jeske, S., Zannini, E., Arendt, E.K., 2017. Evaluation of Physicochemical and Glycaemic Properties of
368 Commercial Plant-Based Milk Substitutes. Plant Foods Hum. Nutr. 72, 26–33.
369 https://doi.org/10.1007/s11130-016-0583-0

370 Katina, K., Heiniö, R.-L., Autio, K., Poutanen, K., 2006. Optimization of sourdough process for
371 improved sensory profile and texture of wheat bread. LWT - Food Sci. Technol. 39, 1189–1202.
372 https://doi.org/10.1016/J.LWT.2005.08.001

373 Katz, K.A., Mahlberg, M.H., Honig, P.J., Yan, A.C., 2005. Rice nightmare: Kwashiorkor in 2
374 Philadelphia-area infants fed Rice Dream beverage. J. Am. Acad. Dermatol. 52, S69–S72.
375 https://doi.org/10.1016/j.jaad.2004.07.056

376 Keogh, M.K., O'Kennedy, B.T., 1998. Rheology of Stirred Yogurt as Affected by Added Milk Fat,
377 Protein and Hydrocolloids. J. Food Sci. 63, 108–112. https://doi.org/10.1111/j.1365-
378 2621.1998.tb15687.x

379 Lustig, R.H., Schmidt, L.A., Brindis, C.D., 2012. Public health: The toxic truth about sugar. Nature 482,
380 27–29. https://doi.org/10.1038/482027a

381 Lynch, K.M., Zannini, E., Coffey, A., Arendt, E.K., 2018. Lactic Acid Bacteria Exopolysaccharides in
382 Foods and Beverages: Isolation, Properties, Characterization, and Health Benefits. Annu. Rev.
383 Food Sci. Technol. 9, 155–176. https://doi.org/10.1146/annurev-food-030117-012537

384 Magaletta, R.L., DiCataldo, S.N., 2009. In vitro method for the detremination of the glycemic index.
385 U.S. Pat. 2009/0004642 A1. US 2009/0004642 A1. https://doi.org/US 2009/0004642 A1

386 Mäkinen, O.E., Uniacke-Lowe, T., O'Mahony, J. a., Arendt, E.K., 2014. Physicochemical and acid
387 gelation properties of commercial UHT-treated plant-based milk substitutes and lactose free
388 bovine milk. Food Chem. 168, 630–638. https://doi.org/10.1016/j.foodchem.2014.07.036

389 MarketsandMarkets, 2017. Dairy Alternatives Market worth 14.36 Billion USD by 2022. URL
390 https://www.marketsandmarkets.com/PressReleases/dairy-alternative-plant-milk-
391 beverages.asp (accessed 1.30.18).

392 Mintel Press Team, 2017. 48% of Germans are reducing their sugar consumption, posing challenges

for the dessert market. URL http://www.mintel.com/press-centre/food-and-drink/48-of-germans-are-reducing-their-sugar-consumption-posing-challenges-for-the-dessert-market (accessed 3.19.18).

Nutrients Review, 2016. Sweeteners: Relative Sweetness, Calories, Glycemic Index - Nutrients Review [WWW Document]. URL https://www.nutrientsreview.com/articles/sweeteners.html (accessed 3.22.18).

Östman, E.M., Nilsson, M., Liljeberg Elmståhl, H.G.M., Molin, G., Björck, I.M.E., 2002. On the Effect of Lactic Acid on Blood Glucose and Insulin Responses to Cereal Products: Mechanistic Studies in Healthy Subjects and In Vitro. J. Cereal Sci. 36, 339–346. https://doi.org/10.1006/JCRS.2002.0469

Otgonbayar, G.-E., Eom, H.-J., Kim, B.S., Ko, J.-H., Han, N.S., 2011. Mannitol production by Leuconostoc citreum KACC 91348P isolated from Kimchi. J. Microbiol. Biotechnol. 21, 968–71.

Peyer, L.C., Zannini, E., Arendt, E.K., 2016. Lactic acid bacteria as sensory biomodulators for fermented cereal-based beverages. Trends Food Sci. Technol. 54, 17–25. https://doi.org/10.1016/j.tifs.2016.05.009

Ruiz Rodríguez, L., Vera Pingitore, E., Rollan, G., Cocconcelli, P.S., Fontana, C., Saavedra, L., Vignolo, G., Hebert, E.M., 2016. Biodiversity and technological-functional potential of lactic acid bacteria isolated from spontaneously fermented quinoa sourdoughs. J. Appl. Microbiol. 120, 1289–1301. https://doi.org/10.1111/jam.13104

Selhub, E.M., Logan, A.C., Bested, A.C., 2014. Fermented foods, microbiota, and mental health: ancient practice meets nutritional psychiatry. J. Physiol. Anthropol. 33, 2. https://doi.org/10.1186/1880-6805-33-2

Sousa, A., Kopf-Bolanz, K.A., Kopf-Bolanz, K., 2017. Nutritional Implications of an Increasing

Consumption of Non - Dairy Plant-Based Beverages Instead of Cow's Milk in Switzerland. Adv. Dairy Res. 5. https://doi.org/10.4172/2329-888X.1000197

Tamang, J.P., Shin, D.-H., Jung, S.-J., Chae, S.-W., 2016. Functional Properties of Microorganisms in Fermented Foods. Front. Microbiol. 7, 578. https://doi.org/10.3389/fmicb.2016.00578

Venn, B.J., Green, T.J., 2007. Glycemic index and glycemic load: measurement issues and their effect on diet–disease relationships. Eur. J. Clin. Nutr. 61, S122–S131. https://doi.org/10.1038/sj.ejcn.1602942

Wisselink, H., Weusthuis, R., Eggink, G., Hugenholtz, J., Grobben, G., 2002. Mannitol production by lactic acid bacteria: a review. Int. Dairy J. 12, 151–161. https://doi.org/10.1016/S0958-6946(01)00153-4

Wolever, T.M.S., Brand-Miller, J.C., Abernethy, J., Astrup, A., Atkinson, F., Axelsen, M., Björck, I., Brighenti, F., Brown, R., Brynes, A., Casiraghi, M.C., Cazaubiel, M., Dahlqvist, L., Delport, E., Denyer, G.S., Erba, D., Frost, G., Granfeldt, Y., Hampton, S., Hart, V. a., Hätönen, K. a., Henry, C.J., Hertzler, S., Hull, S., Jerling, J., Johnston, K.L., Lightowler, H., Mann, N., Morgan, L., Panlasigui, L.N., Pelkman, C., Perry, T., Pfeiffer, A.F.H., Pieters, M., Ramdath, D.D., Ramsingh, R.T., Robert, S.D., Robinson, C., Sarkkinen, E., Scazzina, F., Sison, D.C.D., Sloth, B., Staniforth, J., Tapola, N., Valsta, L.M., Verkooijen, I., Weickert, M.O., Weseler, A.R., Wilkie, P., Zhang, J., 2008. Measuring the glycemic index of foods: Interlaboratory study. Am. J. Clin. Nutr. 87. https://doi.org/87/1/247S [pii]

Zannini, E., Jeske, S., Lynch, K., Arendt, E.K., 2018. Development of novel quinoa-based yoghurt fermented with dextran producer Weissella cibaria MG1. Int. J. Food Microbiol. 268, 19–26. https://doi.org/10.1016/j.ijfoodmicro.2018.01.001

Highlights

- Quinoa served as a good substrate, facilitating the growth of *L. citreum* TR116 and *L. brevis* TR055
- Exogenous amylolytic enzymes in combination with lactic acid fermentation enabled the reduction of glucose by 40%
- Mannitol was produced with high conversion factors from fructose
- Glycaemic load was reduced by more than a third